Is male reproductive senescence minimised in *Mus* species with high levels of sperm competition?

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ABSTRACT

Sperm competition, an evolutionary process in which the spermatozoa of two or more males compete for the fertilisation of the same ovum, gives rise to several morphological and physiological adaptations. Generally, high levels of sperm competition enhances sperm function. In contrast, advanced age is known to lead to reproductive senescence, including a general decline in sperm function. Sperm competition and advanced age may thus have opposing effects on sperm function. Here we tested the hypothesis that the increase in sperm function in species experiencing high levels of sperm competition will counteract the negative effects of advanced age. We measured a comprehensive set of reproductive traits in young and old males in three species of mice of the genus *Mus*, which differ greatly in their levels of sperm competition. Our prediction was that the expression of reproductive senescence will be highest in the species with low levels of sperm competition and lowest in the species with high levels of sperm competition. Surprisingly, we did not find a strong signal of reproductive senescence in any of the three *Mus* species. Overall, our results did not clearly support our hypothesis that high levels of sperm competition minimise the negative effects of aging in sperm function. However, the pattern observed for the percentage of morphologically normal spermatozoa offered some support to this hypothesis.

Keywords: age and reproduction; ATP; reproductive senescence; rodents; sperm abnormalities; sperm competition; sperm function; sperm morphology

INTRODUCTION

Sperm competition occurs when a female mates with two or more males and the spermatozoa of those males compete for the fertilisation of the female's ova (Birkhead & Møller, 1998; Parker, 1970). Sperm competition is a widespread phenomenon and its occurrence leads to several evolutionary adaptations at the behavioural, morphological and physiological levels (Birkhead & Møller, 1998; delBarco-Trillo, Tourmente & Roldan, 2013). In many taxa, high levels of sperm competition are associated with an increase in the production (delBarco-Trillo *et al.*, 2013), storage and allocation of spermatozoa (delBarco-Trillo, 2011; Parker & Pizzari, 2010), as well as with enhanced sperm function (Fitzpatrick *et al.*, 2009; Gomendio *et al.*, 2006; Gómez Montoto *et al.*, 2011a; Kleven *et al.*, 2009; Martín-Coello *et al.*, 2009). For example, high levels of sperm competition in rodents lead to a higher proportion of spermatozoa that are morphologically normal, motile, and capable of reaching and fertilising the ova (Gomendio *et al.*, 2006; Gómez Montoto *et al.*, 2011a), as well as to modifications in sperm dimensions (Gomendio & Roldan, 2008; Tourmente, Gomendio & Roldan, 2011) and sperm energy metabolism (Tourmente *et al.*, 2013; Tourmente *et al.*, 2015b) that may result in improvements in sperm motility.

In contrast to the positive effect of sperm competition on sperm function, advanced age has been reported to lead to reproductive senescence (García-Palomares *et al.*, 2009a; García-Palomares *et al.*, 2009b), particularly having a negative impact on sperm function (Pizzari *et al.*, 2008). A decline in sperm function with age may be due to the accumulation of *de novo* mutations in the male germline that may occur during each cell division (Radwan, 2003), or to an increasingly impaired process of spermatogenesis with advancing age (Johnson & Gemmell, 2012; Pizzari *et al.*, 2008). These processes may be driven or exacerbated by an accumulation of reactive oxygen species and an escalation of oxidative stress with age (Johnson & Gemmell, 2012; Weir & Robaire, 2006), or by reduced efficiency of DNA repair with advancing paternal age (Paul, Nagano & Robaire, 2011; Sloter *et al.*, 2004).

Indeed, across taxa there is strong evidence for a generalised decline in sperm function with age. Such decline may involve: a decline in the number of

79 germinal cells and Sertoli cells in the seminiferous tubules (Dakouane *et al.*, 2005),
80 a reduction in the number of sperm ejaculated (Sasson, Johnson & Brockmann,
81 2012), an increase in sperm abnormalities (Syntin & Robaire, 2001), a decrease in
82 sperm motility (Møller *et al.*, 2009; Wolf *et al.*, 2000), an increase in sperm DNA
83 damage (Harris *et al.*, 2011; Velando *et al.*, 2011), or decreased reproductive
84 success (Dean *et al.*, 2010).
85 Although the positive effect of sperm competition on sperm function is
86 restricted to species experiencing high levels of sperm competition, the negative
87 impacts of advanced age on sperm function can be considered to be similar across
88 phylogenetically related species. Consequently, we hypothesised that the
89 generalised increase in sperm function in species with high levels of sperm
90 competition will diminish the negative impacts of senescence only in such species.
91 That is, in species with high levels of sperm competition, selective pressures on
92 sperm competitiveness may be strong throughout a male's reproductive life, and
93 may reduce the incidence of sperm senescence.
94 To test our hypothesis, we measured a comprehensive set of reproductive
95 traits in young and old males in three species of mice of the genus *Mus* that differ in
96 their levels of sperm competition based on relative testes size: *M. musculus*, *M.*
97 *spretus*, and *M. spicilegus* (delBarco-Trillo *et al.*, 2016). These reproductive traits
98 included the number of spermatozoa in the caudae epididymides, sperm
99 dimensions and morphology, the percentage of spermatozoa with morphological
100 abnormalities, sperm motility and velocity, and ATP content in spermatozoa. A
101 decrease in sperm function can include lower number of stored spermatozoa,
102 shorter spermatozoa, a higher percentage of spermatozoa with morphological
103 abnormalities, lower motility and velocity, and lower ATP content in spermatozoa.
104 According to our hypothesis, we predicted that the decrease in sperm function in
105 old males would be the highest in the species with low levels of sperm competition
106 (*M. musculus*) and the lowest in the species with high levels of sperm competition
107 (*M. spicilegus*).
108
109 **METHODS**
110
111 **Animals**

We used adult males of three species from the genus *Mus* that differ greatly in their levels of sperm competition: *M. musculus*, *M. spretus*, and *M. spicilegus* (n = 11 per species). These three species have been characterized as a good model for studies on sperm competition in rodents, representing low, intermediate and high levels of sperm competition, respectively (Gomendio *et al.*, 2006; Gómez Montoto *et al.*, 2011a). We selected males of two age classes, hereafter referred as "young" and "old" for simplicity. Young males (n = 6 per species) were 4-6 months of age. At this age, mice are no longer juveniles but at the same time they are not old enough to be affected by reproductive senescence. Old males (n = 5 per species) were 24-28 months of age. Males were selected so that ages of young (155.11 ± 37.31 days; mean ± SD) and old animals (769.93 ± 43.96 days) were similar across species. Old males in our study were older than males considered to be senescent in other studies in mice (Anjum *et al.*, 2012; Biddle *et al.*, 1997; Tognetti *et al.*, 2017). We were not able to measure all reproductive traits for all individuals. However, $n \geq 5$ for any species and age class combination.

Adult males were close descendants of animals acquired from the Institut des Sciences de l'Evolution, CNRS- Université Montpellier 2, France, belonging to the following wild-derived strains: *M. musculus*, strain MPB (from Bialowieza, Poland); *M. spretus*, strain SEB (from Barcelona, Spain), and *M. spicilegus*, strain ZRU (from Kalomoyevka, Ukraine). Crossings in our colony were arranged to minimise inbreeding. All males were maintained under standard conditions (14 h light–10 h darkness, 22–24°C, 55-60% relative humidity); with food (rodent chow, Harlan Laboratories; seeds and fresh apple) and water provided ad libitum. All males used in this study were housed individually for at least a month before sampling to eliminate the possibility that males had a different perceived risk of sperm competition.

The research protocol was approved by the Ethics Committee of the Spanish Research Council (CSIC). All procedures were carried out following Spanish Animal Protection Regulation RD53/2013, which conforms to European Union Regulation 2010/63.

Morphological measurements

Males were sacrificed by cervical dislocation, weighed (in g) and measured (body length and tail length; in mm). To evaluate body condition we calculated a body mass index as weight (in g) / length squared (in mm^2) (Labocha, Schutz & Hayes, 2014). Testes were removed and weighed (in g). Relative testes mass (RTS) has been shown to reflect sperm competition levels in rodents (Bryja *et al.*, 2008; Firman & Simmons, 2008; Long & Montgomerie, 2006; Ramm, Parker & Stockley, 2005; Soulsbury, 2010). RTS was calculated using Kenagy and Trombulak´s rodent-specific regression equation: RTS = testes mass / 0.031 × body mass$^{0.77}$ (Kenagy & Trombulak, 1986).

Compared to young mice, old mice had higher body weights (2-way ANOVA: $F_{1,27} = 23.04$, $p < 0.0001$), longer bodies ($F_{1,27} = 7.28$, $p = 0.012$), and longer tails ($F_{1,27} = 30.08$, $p < 0.0001$). Body mass index (used as a measure of body condition) was also higher in old mice than in young mice ($F_{1,27} = 8.83$, $p = 0.006$).

Relative testes size differed among the three species, following the predicted pattern with lowest values in *M. musculus* and highest in *M. spicilegus* (2-way ANOVA: $F_{2,27} = 221.75$, $p < 0.0001$; Table 1, Supporting Information). Relative testes size, however, did not differ between young and old males ($F_{1,27} = 2.51$, $p = 0.13$).

Sperm suspension preparation and sperm measurements

Mature spermatozoa were collected from the caudae epididymides and vasa deferentia, by placing the tissue in a Petri dish containing Hepes-buffered modified Tyrode's medium (mT-H; see Supporting Information for details) prewarmed to 37°C, making several cuts and allowing spermatozoa to swim out for a period of 5 min. After the 5-min swim-out incubation, the sperm suspension was transferred to a prewarmed eppendorf tube. Each sperm suspension was maintained at 37°C until processing. Some samples were assessed immediately (we will refer to this time as "0 h"). Sperm suspensions were also incubated for 3 h at 37°C in mT-H under air, after which samples were taken and some of the sperm parameters were assessed again (we will refer to this time as "3 h"). The duration of incubation (3 h) was selected based on maintenance of sperm motility in vitro in a subset of rodent species, including the three species here studied (Tourmente *et al.*, 2015b). This period of incubation does not result in a complete sperm immobilization in the

177 species with low sperm survival. Moreover, because fertilisation takes place a few
178 hours after copulation in muroid species for which data are available (Suarez *et al.*,
179 1990), our selected incubation time is within physiological time frames.
180 We used a hemocytometer (modified Neubauer chamber) to estimate the
181 total number of spermatozoa stored in the caudae epididymides. To measure
182 sperm linear dimensions, 5 µl of the sperm suspension was smeared onto a slide,
183 fixed with formaldehyde in a phosphate buffer, stained with Giemsa as previously
184 described (Gómez Montoto *et al.*, 2011a), and examined using bright field
185 microscopy. All samples were evaluated and photographed at 1000× magnification
186 for subsequent digitalization using an Eclipse E-600 microscope (Nikon, Tokyo,
187 Japan) with Pan-Fluor optics and a DS5 camera (Nikon, Tokyo, Japan).
188 Spermatozoa were photographed by using the software NIS-Elements v.3.0 (Nikon,
189 Tokyo, Japan). For each individual, we measured 25 different spermatozoa. Linear
190 dimensions were obtained by measuring captured sperm images using ImageJ
191 software v.1.41 (National Institutes of Health, Bethesda, MD, USA) (Gómez
192 Montoto *et al.*, 2011b). Measurements included head length, head width, head area,
193 total flagellum length, and total sperm length. Head length was measured as the
194 linear distance between the most basal point and the most apical one of the sperm
195 head. Head width was taken as a straight line between the dorsal and ventral
196 regions in the wider region of the sperm head. Head area was measured
197 considering the entire sperm head including the apical hook.
198 To quantify differences in sperm head morphology we used a geometric
199 morphometric approach described previously (Varea Sánchez, Bastir & Roldan,
200 2013). See Supporting Information for details.
201 To assess sperm abnormalities, we used sperm smears stained first with
202 eosin-nigrosin and subsequently with Giemsa (Gómez Montoto *et al.*, 2011a).
203 Briefly, 5 µl sperm suspension and 10 µl eosin-nigrosin solution were mixed on a
204 glass slide placed on a stage at 37°C and 30 s later the mix was smeared and
205 allowed to air-dry. Smears were stained with Giemsa solution and mounted with
206 DPX. Smears were examined at 1000x under bright field and 200 spermatozoa per
207 male were examined to evaluate the percentage of morphologically normal
208 spermatozoa (i.e. without abnormal head, midpiece or principal piece, and without
209 a cytoplasmic droplet or coiled flagella).

The percentage of motile spermatozoa (MOT) was evaluated by examining 10 µl of the sperm suspension that was placed between a pre-warmed slide and a coverslip at 100× magnification under phase contrast optics. We also estimated the percentage of spermatozoa exhibiting forward progression. To assess sperm swimming patterns, an aliquot of sperm suspension was diluted to approximately 5×10^6 spermatozoa ml^{-1}, placed in a pre-warmed microscopy chamber with a depth of 20 µm (Leja, Nieuw-Vennep, The Netherlands), and filmed at 40× using a phase contrast microscope connected to a digital video camera (Basler A312fc, Vision Technologies, Glen Burnie, MD). A minimum of 150 sperm trajectories were assessed per male using a computer-assisted sperm analyzer (CASA; Sperm Class Analyzer version 4.0, Microptic, Barcelona, Spain), and the following swimming parameters were estimated for each trajectory: curvilinear velocity (VCL, µm s^{-1}), straight line velocity (VSL, µm s^{-1}), average path velocity (VAP, µm s^{-1}), wobble (WOB = VAP/VCL), linearity (LIN = VSL / VCL), straightness (STR = VSL / VAP), amplitude of lateral head displacement (ALH, µm), and beat-cross frequency (BCF, Hz).

Sperm ATP content was measured using a luciferase-based ATP bioluminescence assay kit (HS II, Roche Applied Science) (Tourmente *et al.*, 2015a). See Supporting Information for details.

Statistical analyses

All statistical analyses were conducted using R version 3.1.0 (R Core Team, 2014) unless otherwise specified. Normality was checked with the Shapiro-Wilk normality test. If normality was not met, we used logarithmic and arcsine transformations as required. Average values are reported as mean ± SD. Significance level (α) was set at 0.05 for all the tests.

We used principal component analysis (PCA) to reduce potentially correlated variables and obtain measures of "overall sperm morphology", "overall sperm velocity", and "overall trajectory shape". See Supporting Information for details.

We implemented 2-way ANOVAs and 2-way ANCOVAs fitted using the function *aov*. The two factors were species (3 levels) and age (young and old). The covariate in the ANCOVAs was body mass. We also considered the interaction between species and age to determine if any significant difference between young

and old mice differed across species, and whether such species effect paralleled the different levels of sperm competition among those species.

Geometric morphometrics statistical analyses were conducted with MorphoJ v1.06d (Klingenberg, 2011). Differences in sperm head shape between young and old individuals were quantified by examining the distance between the mean of both groups conducting a discriminant analysis (Timm, 2002).

RESULTS

The number of spermatozoa stored in the cauda epididymides differed between species, following the predicted pattern (2-way ANCOVA: $F_{2,26} = 50.36$, $p < 0.0001$; Table 1, Supporting Information), but there was no difference between young and old males ($F_{1,26} = 0.4$, $p = 0.54$).

The overall sperm morphology differed between species ($F_{2,24} = 168.42$, $p > 0.0001$), and between young and old males ($F_{1,24} = 6.15$, $p = 0.02$), but there was no significant interaction between species and age ($F_{2,24} = 0.1$, $p = 0.91$). The shape of the sperm head differed between species (discriminant analyses: $p < 0.0001$), but not between young and old mice ($p = 0.2$; Fig. 1).

The percentage of normal spermatozoa differed between species ($F_{2,27} = 7.44$, $p = 0.003$); even though there was not an overall difference between young and old males ($F_{1,27} = 0.99$, $p = 0.33$), we found a statistically significant interaction between species and age ($F_{2,27} = 4.07$, $p = 0.029$). Subanalyses by species showed no differences between young and old mice in *M. spicilegus* and *M. spretus* ($p > 0.05$) but a higher percentage of normal spermatozoa in young males than in old males in *M. musculus* ($p = 0.008$; Fig. 2), the species with the lowest level of sperm competition.

There were differences in sperm motility across species (2-way ANCOVA: $F_{2,26} = 36.47$, $p < 0.0001$), this trait being higher in young males than in old males ($F_{1,26} = 7.25$, $p = 0.01$). However, all species were affected similarly by age (interaction: $F_{2,26} = 0.1$, $p = 0.9$). After 3 hours of incubation, significant differences among species remained in sperm motility ($F_{2,26} = 25.05$, $p < 0.0001$) but there were no longer differences between age classes ($F_{1,26} = 0.41$, $p = 0.53$). Sperm forward progression also differed between species, both at 0 h ($F_{2,26} = 6.22$, $p = 0.006$) and after 3 hours of incubation ($F_{2,26} = 17.71$, $p < 0.0001$), and while it was

similar in young and old males at 0 h ($F_{1,26}$ = 0.25, p = 0.62), after 3 hours of incubation it was higher in old males than in young males ($F_{1,26}$ = 7.38, p = 0.01).

Overall sperm velocity and overall trajectory shape differed between species (p < 0.0001 in both analyses) and ages (p < 0.01) but there was not a significant interaction between species and age (p > 0.05).

There were significant differences between species in the amount of ATP per sperm cell, both at 0 h ($F_{2,26}$ = 6.85, p = 0.004) and after 3 hours of incubation ($F_{2,26}$ = 9.59, p = 0.0008; Table 1, Supporting Information). However, there was not a significant difference in ATP concentration between young and old males, nor a significant interaction between species and age at either time (p > 0.05 for all analyses).

DISCUSSION

Overall, our results do not support the hypothesis that males in species with high levels of sperm competition suffer less reproductive senescence than in species with low levels of sperm competition. We found that many reproductive traits were unaffected by age, whereas others were either enhanced or lessened in old males compared to young males, but the level of sperm competition did not have an influence in most of these traits. Only the percentage of normal spermatozoa matched our prediction, with a decline in old males in *M. musculus* (i.e. the species with low levels of sperm competition) but not in the other two species, which experience higher levels of sperm competition (*M. spretus* and *M. spicilegus*). This result may be driven by an enhanced process of spermatogenesis in species with high levels of sperm competition, which would either directly or indirectly minimise the occurrence of sperm abnormalities in old males, but this is an area of research that requires further investigation.

Even though we considered many reproductive traits that could be affected by senescence, there are many other traits that could be differently affected in young and old males of a species depending on the level of sperm competition normally experienced in that species. These traits include chromosomal abnormalities, DNA damage in spermatozoa, and any traits that regulate or determine the success of the capacitation and fertilisation processes (Gogol,

309 Bochenek & Smorag, 2002; Momand, Xu & Walter, 2013). For example, in brown
310 rats, the spermatozoa of old males are more susceptible to oxidative damage and
311 DNA fragmentation (Zubkova, Wade & Robaire, 2005), as well as having a
312 decreased antioxidant capacity and an increased production of reactive oxygen
313 species (Weir & Robaire, 2006). It is important to notice that the genomic damage
314 in spermatozoa driven by aging may be independent of sperm function. Despite a
315 normal expression of sperm function in old males, any genomic damage in their
316 spermatozoa will increase the risk of transmission of multiple genetic and
317 chromosomal defects to offspring (Wyrobek *et al.*, 2006).
318 Surprisingly, we did not find a strong signal of reproductive senescence in
319 the three species of mice that we studied. Reproductive senescence may thus not
320 play an important role in the natural populations of the three *Mus* species under
321 study. Indeed, given the high predation rates suffered by rodents, most males will
322 normally die before the inception of any signs of senescence. Another study using
323 wild-captured *Mus musculus domesticus* found that epididymal sperm counts
324 declined with age, although only a range of relatively advanced ages (21-32
325 months) were studied (Garratt *et al.*, 2011). It must be noted that most of the
326 available knowledge on reproductive senescence in rodents is based on laboratory
327 strains (Katz-Jaffe *et al.*, 2013; Lucio *et al.*, 2013; Parkening, 1989). This may be a
328 shortcoming, as in a benign captive environment the negative effects of aging can
329 be minimized and thus differences between age classes might be obscured.
330 Even though there are many studies describing the timing and incidence of
331 reproductive senescence, there are also many studies in which reproductive
332 condition remains unchanged or is even enhanced in old individuals (Gasparini *et*
333 *al.*, 2010; Johnson & Gemmell, 2012; Kanuga *et al.*, 2011). It is still unclear why
334 reproductive senescence is pronounced in some species but not in others.
335 It is important to consider whether any differences between young and old
336 males lead to a fertilising advantage for one age type or the other. For example,
337 young male guppies produce faster-swimming spermatozoa compared to old
338 males; however, young males do not have a fertilising advantage under sperm
339 competition scenarios (Gasparini *et al.*, 2010). It is equally possible that despite a
340 lack of consistent differences in sperm function between young and old males, as
341 we found in our three species of mice, undetected differences between their

spermatozoa could result in a lower reproductive potential in older individuals. We can thus conclude that even though our results did not support our hypothesis that high levels of sperm competition can minimise the impacts of senescence, more reproductive measurements, including sperm competition tests, and measurements of fertilising ability and offspring health, and possibly higher sample sizes than we used, are required to fully support or disprove our hypothesis.

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FIGURE LEGENDS

Figure 1. Sperm head shape in young and old males of three *Mus* species. Dots indicate the landmarks used for geometric morphometrics analyses.

Figure 2. Percentage of morphologically normal spermatozoa in young and old males of three *Mus* species. For each boxplot, the bar within each box represents the sample median, each box represents 50% of the data around the median, and the two whiskers represent the 95% confidence interval. ** denotes $p < 0.001$; NS denotes $p > 0.05$.